Synthesis of Destetrapeptide B^{27-30} Human (Porcine) Insulin. A Biologically Active Insulin Analog[1,2]

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Abstract: The synthesis and isolation in purified form of a biologically active analog of human (porcine) insulin are described. This analog differs from the parent compound in that the C-terminal tetrapeptide sequence of the B chain moiety of the insulin molecule has been eliminated. For the synthesis of this analog, the destetrapeptide B^{27-30} chain of human insulin was chemically synthesized by the fragment condensation method and isolated as the S-sulfonated derivative. Combination of the latter compound with the sulfhydryl form of the A chain of human (porcine) insulin afforded the destetrapeptide B^{27-30} human (porcine) insulin. The insulin analog was separated from the unreacted chains and the by-products found in the combination mixture and obtained in purified form by chromatography on a carboxymethylcellulose column with an exponential sodium chloride gradient. The destetrapeptide B^{27-30} insulin possesses a potency of 11–15 IU/mg when assayed by the mouse convulsion method and 7 IU/mg by the radioimmunoassay technique. It is concluded that the C-terminal tetrapeptide sequence of the B chain of insulin is not critically involved in the manifestation of the biological properties of the hormone.

Studies have been initiated in our laboratory directed toward the elucidation of any possible correlation between chemical structure and biological activity of insulin; Figure 1 illustrates the structure of human insulin with appropriate numbering of the constituent amino acid residues. To this end a number of insulin analogs have been synthesized and tested for biological activity.[3-6] One of these synthetic analogs, the destripeptide B^{28-30} insulin, differs from the parent molecule in that the C-terminal tripeptide sequence of the B chain (sequence B^{28}–B^{30}) has been eliminated and the newly exposed C-terminal amino acid residue threonine (B^{27}) has been converted to the corresponding amino alcohol and amino aldehyde derivatives.[3] Biological evaluation has shown that this analog possesses a potency equivalent to that of insulin when tested by the mouse convulsion assay method. This finding demonstrates that the three amino acid residues occupying the C-terminal sequence of the B chain in the parent molecule are not necessary for the biological activity of insulin. It also infers that an intact amino acid residue at position B^{27} (threonine) is not a requirement for a fully active hormone molecule.[3] The X-ray analysis of the three-dimensional structure of insulin has shown that the amino acid residue, tyrosine, located at B^{26} is part of a nonpolar area on the surface of the hormone molecule.[7,8] It was further shown that this nonpolar area is adjacent to the region which the existing evidence suggests that it might be the "active center" of insulin. This region includes the amino and carboxyl terminal segments of the A chain and the B chain segment involving sequences B^{22} to B^{24} or B^{25}. The amino acid residues found in the aforementioned nonpolar and "active center" regions of insulin are invariant in most species studied thus far.[9]

In view of these considerations, it was of interest to find what effect the elimination of the B^{27} amino acid residue and the termination of the B chain, with the B^{26} residue bearing a free carboxyl group, will have on the biological activity of insulin. The present communication describes the synthesis, isolation in pure form, and biological evaluation of such an analog of human (porcine) insulin. This analog, the destetrapeptide B^{27-30} insulin, differs from the parent molecule in that the C-terminal tetrapeptide sequence of the B chain (sequence B^{27}–B^{30}) has been eliminated. By the mouse convulsion assay method, this analog was found to possess a specific activity ranging from 11 to 15 IU as compared with the specific activity of 23–25 IU found in our laboratory for the natural insulin. By the radioimmunoassay method, the destetrapeptide B^{27-30} insulin possesses a potency of 7 IU/mg. It thus becomes apparent that elimination of the C-terminal tetrapeptide sequence of the B chain moiety of insulin (sequence B^{27}–B^{30}) and termination of that chain with the B^{26} residue, tyrosine, bearing a free carboxyl group, results in approximately 50% loss of the biological activity of the hormone. On the other hand, as was mentioned earlier, termination of the B-chain moiety of insulin with a modified B^{27} amino acid residue (elimination of sequences B^{28}–B^{30}) does not affect the biological activity of the hormone.[3] These data indicate that the C-terminal tetrapeptide segment (B^{27}–B^{30}) of the B chain of insulin does not participate functionally in the mechanism of action of the hormone, although the presence of an amino alcohol or amino aldehyde derivative of threonine at position B^{27} confers full activity on the molecule. The question arises whether the full activity is directly dependent on the

(1) This work was supported by the National Institute for Arthritis and Metabolic Diseases, U. S. Public Health Service (AM 12925).

(2) For the previous paper of this series, see P. G. Katsoyannis, Y. Okada, and C. Zalut, *Biochemistry*, **2**, 2516 (1973).

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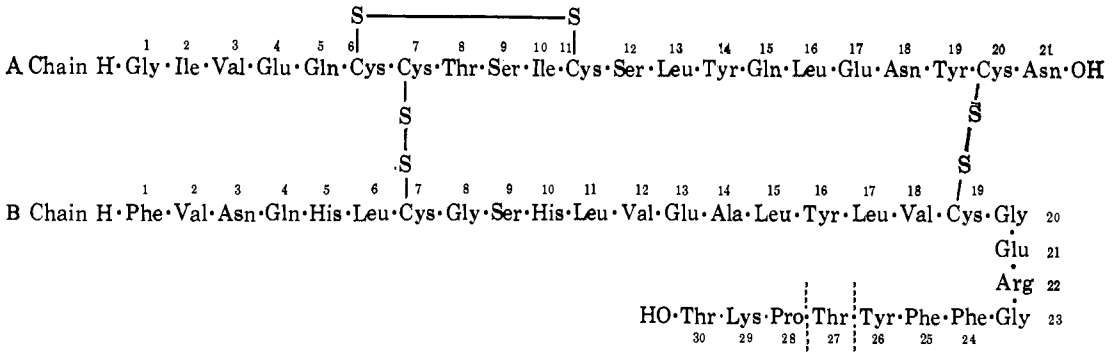
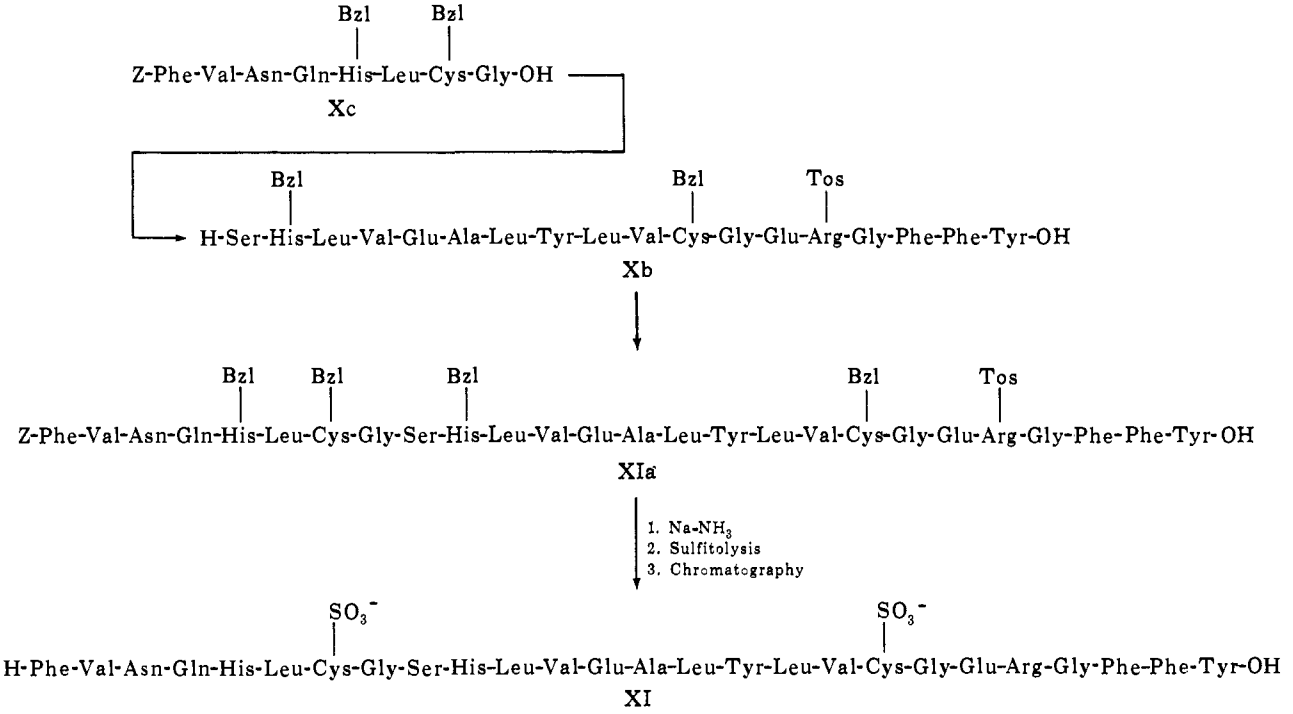


Figure 1. Structure of human insulin. Dotted lines indicate the destripeptide B^{28-30} and destetrapeptide B^{27-30} insulins.

Chart I



physical presence of the modified threonine residue (B^{27}) or is simply due to the absence of a negative charge. It may well be that the drop in biological activity of the destetrapeptide B^{27-30} insulin is a result of the presence of a carboxylate anion (B^{26}) in the nonpolar area adjacent to the hypothetical active region of the hormone. Work is now in progress to clarify these possibilities. The more pronounced loss of the immunological activity of this analog as measured by the radioimmunoassay method in comparison with the loss of its biological activity is another example of the ill-defined interdependency between sites responsible for physiological and immunological reactivity of insulin.[10]

General Aspects of the Synthesis of the Human (Porcine) Insulin Analog. The destetrapeptide B^{27-30} human (porcine) insulin was prepared by the combination of the sulfhydryl form of the human (porcine) A chain with the S-sulfonated form of the destetrapeptide B^{27-30} chain. The sulfhydryl form of the A chain of human insulin, which is identical with the respective chain of porcine insulin,[11] was prepared by reduction with 2-mercaptoethanol of the S-sulfonated derivative of that chain. The latter compound was prepared by oxidative sulfitolysis of porcine insulin followed by separation of the resulting S-sulfonated derivatives of the A and B chains by continuous flow electrophoresis. The sulfitolysis of insulin, the separation of the resulting A and B chain derivatives, and the conversion of the S-sulfonated A chain to its sulfhydryl form have been described in detail in previous communications from this laboratory.[12,13] The synthesis of the S-sulfonated destetrapeptide B^{27-30} chain was patterned after that of the natural sheep and human B chains.[14-16] It involved the construction of the protected hexacosapeptide XIa (Chart I), removal of the protecting groups

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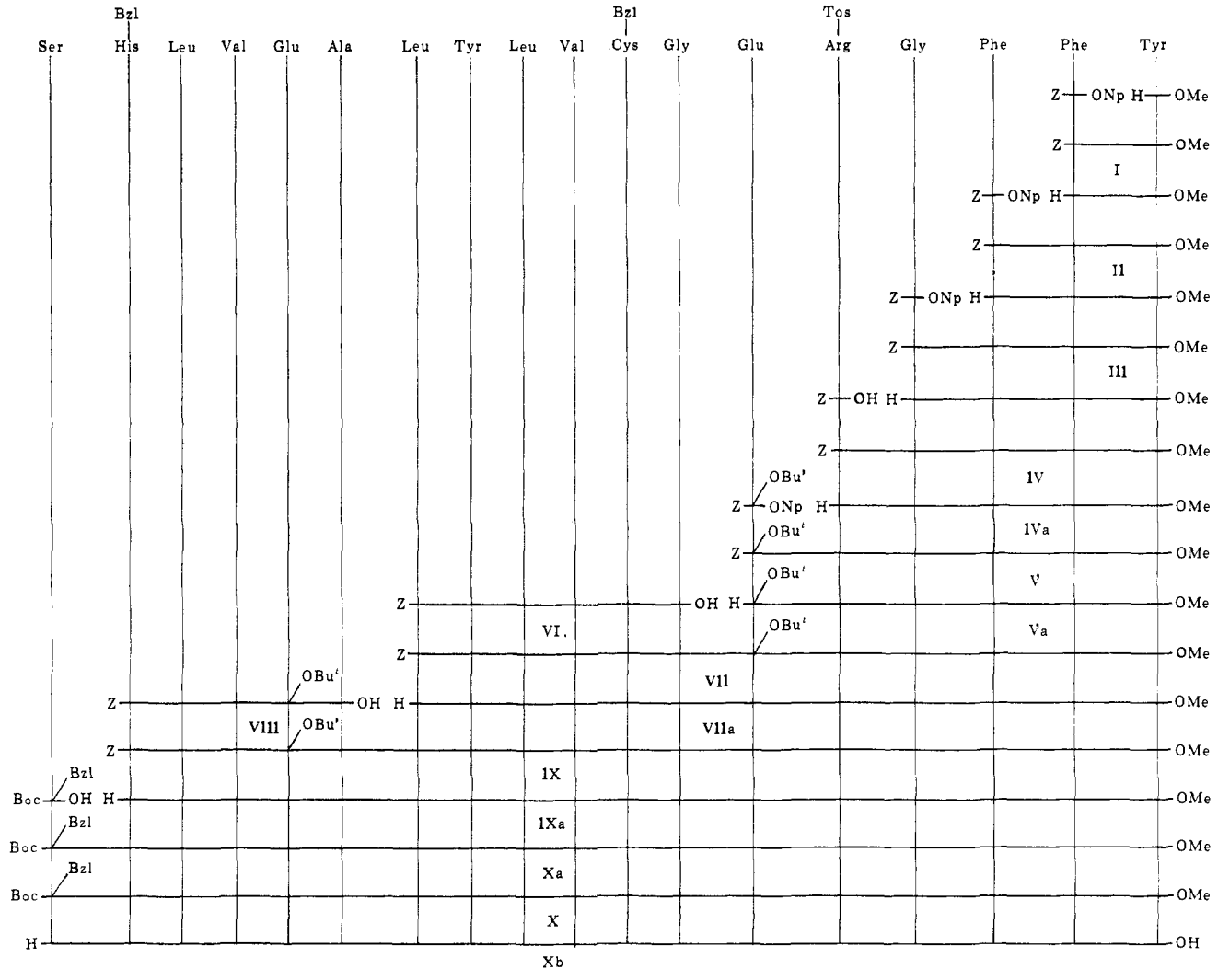
Chart II



with sodium in liquid ammonia,[17] and conversion of the resulting sulfhydryl derivative to the S-sulfonated form. The synthesis of the protected hexacosapeptide XIa was accomplished by the coupling of the N-terminal octapeptide fragment[18] Xc with the C-terminal octadecapeptide derivative Xb by the *N,N'*-dicyclohexylcarbodiimide–*N*-hydroxysuccinimide method.[19] For the construction of the partially protected octadecapeptide derivative Xb (Chart II), we have employed the fragment condensation approach. Peptide fragments were prepared stepwise and were condensed to form larger peptide subunits. Protection of the amino function was effected by the use of the benzyloxycarbonyl group and in one instance by the use of the *tert*-butoxycarbonyl group. Activation of the acylated amino acids used at each synthetic step was brought about in all but two instances by conversion to the corresponding *p*-nitrophenyl esters. Activation of N^{α}-benzyloxycarbonyl-N^{ω}-tosyl-L-arginine[20] in the synthesis of the pentapeptide derivative IV and of *N-tert*-butoxycarbonyl-*O*-benzyl-L-serine[21] in the synthesis of the octadecapeptide derivative Xa was accomplished by the use of 2-ethyl-5-phenylisoxazolium 3'-sulfonate[22] and *N,N'*-dicyclohexylcarbodiimide-*N*-hydroxysuccinimide, respectively. Peptide bond formation in the "fragment condensation" was carried out exclusively by the *N,N'*-dicyclohexylcarbodiimide–*N*-hydroxysuccinimide method.[19] Removal of the benzyloxycarbonyl group at various synthetic steps was accomplished by catalytic hydrogenation or on exposure to hydrogen bromide in trifluoroacetic acid and of the *tert*-butoxycarbonyl group on exposure to trifluoroacetic acid.

Decarbobenzoxylation of the pentapeptide fragment IV and coupling of the ensuing partially protected derivative IVa with *N*-benzyloxycarbonyl-L-glutamic acid γ-*tert*-butyl-α-*p*-nitrophenyl ester[23] afforded the fully protected hexapeptide V. Catalytic hydrogenation of the latter compound and condensation of the resulting peptide derivative Va with the partially protected hexapeptide fragment VI afforded the fully blocked dodecapeptide fragment VII. On exposure to hydrogen bromide in trifluoroacetic acid the benzyloxycarbonyl and *tert*-butyl ester groups were removed from peptide VII and the resulting partially protected derivative VIIa was condensed with the pentapeptide

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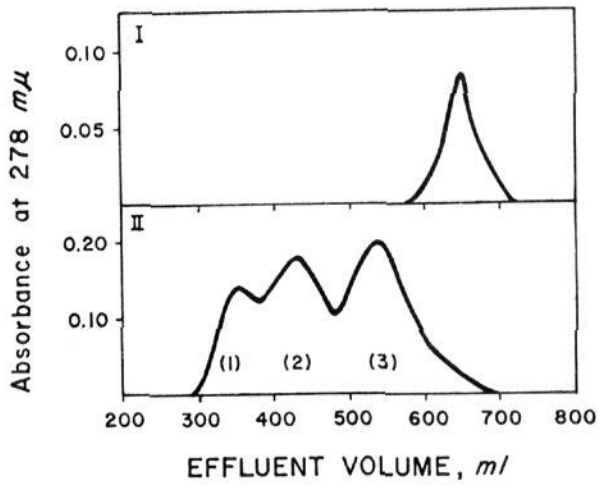

Figure 2. Elution patterns from chromatography on a 4 × 55 cm *CM-cellulose column with urea–acetate* buffer, pH 4.0: (I) natural bovine insulin B chain *S*-sulfonate; (II) crude mixture obtained by the sodium in liquid ammonia treatment of synthetic destetrapeptide B^{27-30} chain followed by sulfitolysis, dialysis, and lyophilization.

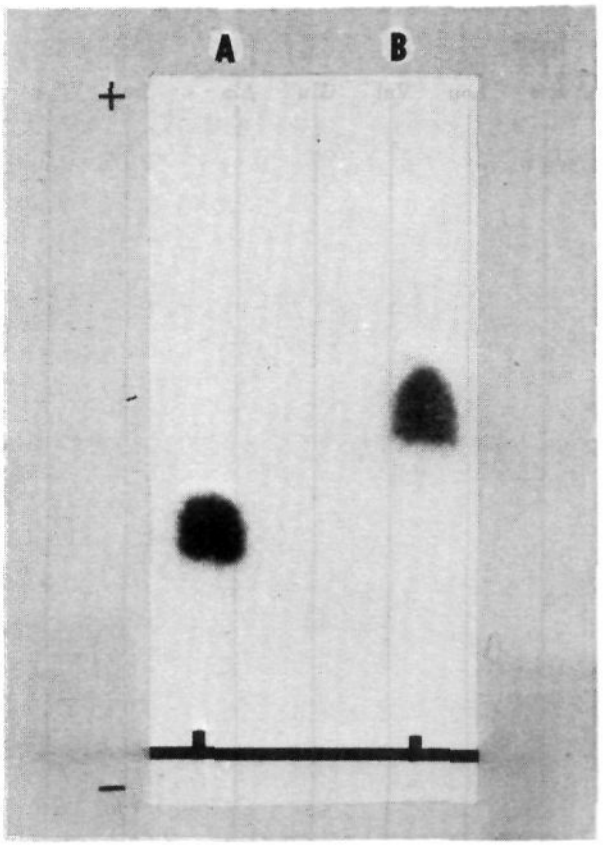

Figure 3. High voltage thin-layer electrophoresis of natural bovine insulin B chain *S*-sulfonate (A) and synthetic B^{27-30} chain *S*-sulfonate (B): 0.01 *M* NH_4HCO_3 adjusted to pH 10.0 with NH_4OH, 2800 V, 25 min.

fragment VIII to give the heptadecapeptide IX. The subsequent step consisted in the deprotection of the heptadecapeptide IX with hydrogen bromide in trifluoroacetic acid and the condensation of the resulting derivative IXa with *N-tert*-butoxycarbonyl-*O*-benzyl-L-serine. The ensuing protected octadecapeptide Xa upon treatment with NaOH was converted to derivative X which on exposure to trifluoroacetic acid afforded the octadecapeptide Xb bearing free α-amino and carboxyl groups. In a final step (Chart I), the N-terminal octapeptide fragment Xc was condensed with the C-terminal partially protected octadecapeptide fragment Xb to give the protected hexacosapeptide XIa.

Removal of benzyloxycarbonyl, benzyl, and *p*-toluenesulfonyl blocking groups from the protected hexacosapeptide XI was accomplished by sodium in liquid ammonia in the glass apparatus which we have devised for this type of reaction.[16] The reduced product was dissolved in 8 *M* guanidine hydrochloride and subjected to oxidative sulfitolysis upon treatment with sodium sulfite and sodium tetrathionate at pH 8.9 upon which the sulfhydryl groups were converted to the *S*-sulfonates.[15,16] Dialysis of the reaction mixture followed by lyophilization of the dialysate afforded the crude destetrapeptide B^{27-30} chain *S*-sulfonate. For the purification of this material, we have followed the procedure we have developed for the purification of human and sheep insulin B chains.[15,16] The lyophilized material was thus chromatographed on a carboxymethylcellulose (CM-cellulose) column equilibrated and eluted with a urea–acetate buffer, pH 4.0. From the elution pattern of this column, shown in Figure 2-II, it is apparent that at least three major components (1, 2, and 3) are present. Identification of components 1 and 2 was not attempted. Component 3 proved to be the destetrapeptide B^{27-30} chain *S*-sulfonate. As was expected, this B-chain analog, which lacks a lysine residue, is eluted earlier from the CM-cellulose column than the intact natural B chain *S*-sulfonate (Figure 2-I). For the isolation of component 3 from the urea-containing chromatographic effluent, the fractions under peak 3 were pooled and passed through a Sephadex G-15 column using 5% acetic acid as the eluting solvent. Further purification of the B-chain analog was accomplished by its conversion to the picric acid salt and chromatography on a Sephadex G-15 column equilibrated and eluted with ammonium bicarbonate buffer. Probably due to the absence of a lysine residue, the picrate of the B-chain analog, in contrast to that of the intact natural B chain, has some solubility in water. We have, therefore, encountered considerable losses in the isolation of this analog.

Amino acid analysis of the purified material after acid hydrolysis gave a composition expressed in molar ratios in good agreement with the theoretically expected values (Table I). Digestion of this material with aminopeptidase M and amino acid analysis of the digest showed that the constituent amino acids were present in the theoretically expected ratios for the destetra-

Table I. Amino Acid Composition[a] of an Acid Hydrolysate and an APM Digest of the S-Sulfonated Destetrapeptide B^{27-30} Chain of Insulin

Amino acid	Acid hydrolysis		Enzymatic hydrolysis (APM)	
	Theory	Found	Theory	Found
Histidine	2.0	2.0	2.0	1.9
Arginine	1.0	1.0	1.0	0.9
Aspartic acid	1.0	1.1	0	0
Glutamine	0	0	1	2.2[b]
Asparagine	0	0	1	
Serine	1.0	0.9	1.0	1.0[c]
Glutamic acid	3.0	3.0	2.0	2.0
Glycine	3.0	3.2	3.0	2.9
Alanine	1.0	1.0	1.0	1.2
Half-cystine	2.0	1.2	0	0
Valine	3.0	2.7	3.0	3.0
Leucine	4.0	3.8	4.0	4.3
Tyrosine	2.0	1.3	2.0	1.8
Phenylalanine	3.0	2.9	3.0	2.8
S-Sulfocysteine	0	0	2.0	2.2[d]

[a] Number of amino acid residues per molecule. [b] Emerge on the same position. [c] Separated from glutamine and asparagine in a 30° chromatographic run. [d] Eluted from the long column of the Beckman-Spinco analyzer after 26 ml of effluent.

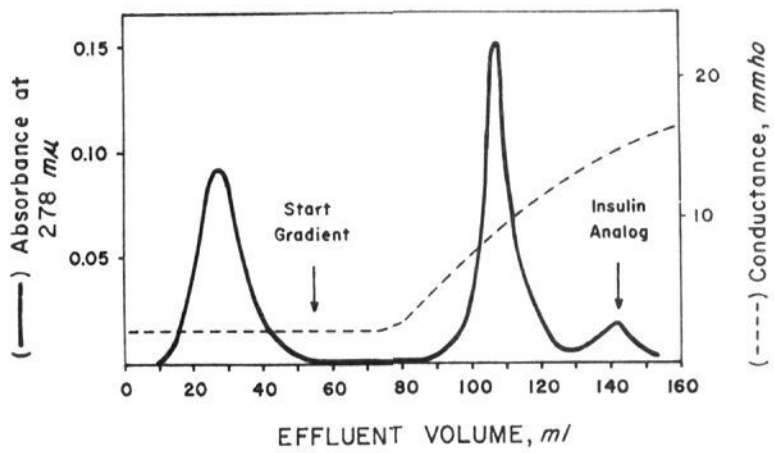

Figure 4. Chromatography of a combination mixture of the sulfhydryl form of human (porcine) insulin A chain with destetrapeptide B^{27-30} chain *S*-sulfonate on a 0.9 × 23 cm CM-cellulose column with acetate buffer (pH 3.3; $[Na^+]$ 0.024 *M*) and an exponential NaCl gradient. Two combination mixtures (see Experimental Section) were processed. The column effluent was monitored by a Gilford recording spectrophotometer and by a conductivity meter (Radiometer, Copenhagen). The destetrapeptide B^{27-30} human (porcine) insulin (130–150 ml of effluent) was recovered as the hydrochloride.

peptide B^{27-30} chain and thus established the stereochemical homogeneity of the synthetic product within the limits of error of the enzymatic technique (Table I). On high voltage thin-layer electrophoresis and at pH 10, the synthetic truncated chain moved as a single component (Pauly reaction) and as was expected had a greater mobility toward the anode than the natural B chain *S*-sulfonate (Figure 3).

Isolation of the Insulin Analog. The conversion of the S-sulfonated A chain of human (porcine) insulin to its sulfhydryl form and its combination with the destetrapeptide B^{27-30} chain to produce the truncated insulin was accomplished according to the procedure developed in this laboratory and successfully used in the synthesis of human and sheep insulins.[13,24,25] As was the case with the latter two insulins, isolation of the synthetic analog in a highly purified form from the combination mixture of its two chains was performed by chromatography on a CM-cellulose column using an acetate buffer (pH 3.3) and an exponential NaCl gradient.[13,25] The chromatographic pattern obtained is shown in Figure 4. The insulin analog is eluted with application of the NaCl gradient and is the slowest moving component. The material eluted just before the insulin analog was shown to be the unreacted destetrapeptide B^{27-30} chain. An identical situation exists with the chromatographic pattern of natural insulin, all synthetic and half-synthetic insulins.[25] From the effluent of the column, the insulin analog was isolated *via* picrate as the hydrochloride by a procedure that we have reported previously.[13,25]

Amino acid analysis of the destetrapeptide B^{27-30} insulin after acid hydrolysis gave a composition expressed in molar ratios in agreement with the theoretically expected values (Table II). On thin-layer electrophoresis in 0.5 *N* acetic acid the synthetic analog moved as a single component (Pauly reaction) and had a mobility slightly different than the natural insulin (Figure 5).

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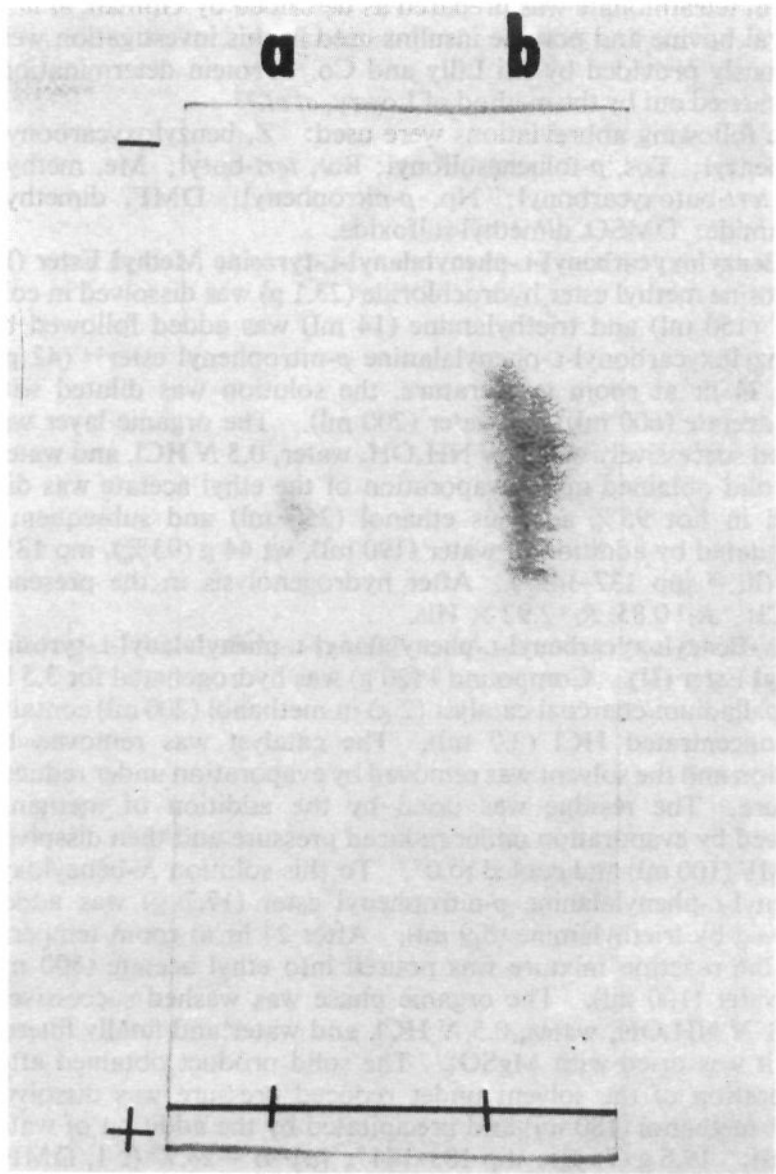

Figure 5. High voltage thin-layer electrophoresis of synthetic destetrapeptide B^{27-30} human (porcine) insulin (A) and natural bovine insulin (B): 0.5 *N* acetic acid, pH 2.9, 3400 V, and 15 min.

Experimental Section

Melting points for all compounds were taken in capillary tubes and are not corrected. For paper chromatography, the protected peptides were deblocked as indicated in the experimental part and the resulting compounds were chromatographed on Whatman No. 1 filter paper at room temperature. R_f^1 values refer to the Partridge system;[26] R_f^2 values refer to the system[27] 1-butanol pyridine acetic acid–water, 30:20:6:24, and are expressed as a multiple of the distance traveled by a histidine marker. Optical rotations were taken with a Zeiss photoelectric precision polarimeter. The amino acid analyses were performed with a Beckman–Spinco amino acid analyzer (Model 120C) equipped with a digital readout system (Model CRS 12AB, Infotronics Corp., Houston, Texas) according to the method of Spackman, *et al.*[28] Acid hydrolysis and calculations of molar ratios were carried out as described previously.[12] For the enzymatic digestion with aminopeptidase M (APM) the method of Pfleiderer, *et al.*,[29] was employed. APM was purchased from Henley and Co., New York, N. Y. Thin-layer electrophoresis was carried out according to a method developed in this laboratory[30] and was performed with a Wieland-Pfleiderer pherograph (Brinkman Instruments). Preswollen microgranular CM-cellulose (Whatman CM 52/1) and Sephadex G-15 (Pharmacia Uppsala) were used in this investigation. The washing of the resins and the preparation of the columns and of the buffers used were described in a previous report.[12,13] Biological assays were carried out by the mouse convulsion method as has been described previously.[24,25] Radioimmunoassays were performed by the method of Hales and Randle[31] using an insulin immunoassay kit (Amersham/Searle Co.).

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Sodium tetrathionate was prepared as described by Gilman, *et al.*[32] Natural bovine and porcine insulins used in this investigation were generously provided by Eli Lilly and Co. Protein determinations were carried out by the method of Lowry, *et al.*[33]

The following abbreviations were used: Z, benzyloxycarbonyl; Bzl, benzyl; Tos, *p*-toluenesulfonyl; Bu^t, *tert*-butyl; Me, methyl; Boc, *tert*-butoxycarbonyl; Np, *p*-nitrophenyl; DMF, dimethylformamide; DMSO, dimethyl sulfoxide.

N-Benzyloxycarbonyl-L-phenylalanyl-L-tyrosine Methyl Ester (I). L-Tyrosine methyl ester hydrochloride (23.1 g) was dissolved in cold DMF (150 ml) and triethylamine (14 ml) was added followed by *N*-benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester[34] (42 g). After 24 hr at room temperature, the solution was diluted with ethyl acetate (600 ml) and water (200 ml). The organic layer was washed successively with 1 *N* NH_4OH, water, 0.5 *N* HCl, and water. The solid obtained upon evaporation of the ethyl acetate was dissolved in hot 95% aqueous ethanol (250 ml) and subsequently precipitated by addition of water (190 ml), wt 44 g (93%), mp 135–138° (lit.[35] mp 137–149°). After hydrogenolysis in the presence of HCl: R_f^1 0.85, R_f^2 2.92 × His.

N-Benzyloxycarbonyl-L-phenylalanyl-L-phenylalanyl-L-tyrosine Methyl Ester (II). Compound I (20 g) was hydrogenated for 3.5 hr over palladium/charcoal catalyst (2 g) in methanol (200 ml) containing concentrated HCl (3.7 ml). The catalyst was removed by filtration and the solvent was removed by evaporation under reduced pressure. The residue was dried by the addition of methanol followed by evaporation under reduced pressure and then dissolved in DMF (100 ml) and cooled to 0°. To this solution *N*-benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester (17.7 g) was added followed by triethylamine (5.9 ml). After 24 hr at room temperature, the reaction mixture was poured into ethyl acetate (300 ml) and water (100 ml). The organic phase was washed successively with 1 *N* NH_4OH, water, 0.5 *N* HCl, and water and finally filtered after it was dried with $MgSO_4$. The solid product obtained after evaporation of the solvent under reduced pressure was dissolved in hot methanol (180 ml) and precipitated by the addition of water (70 ml): 19.6 g (75%); mp 183–184°; $[\alpha]^{26}D$ −23.7° (*c* 1, DMF). After hydrogenolysis in the presence of HCl: R_f^1 0.88, R_f^2 3.54 × His.

Anal. Calcd for $C_{36}H_{37}N_3O_7$: C, 69.3; H, 5.97; N, 6.7. Found: C, 69.4; H, 6.11; N, 7.0.

N-Benzyloxycarbonylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosine Methyl Ester (III). Compound II (15.75 g) was hydrogenated for 3.5 hr over palladium/charcoal catalyst (2 g) in methanol (250 ml) containing concentrated HCl (2.2 ml). The catalyst was filtered off, and the filtrate was concentrated to dryness under reduced pressure. To a solution of the residue in DMF (100 ml) cooled to 0°, *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester[36] (8.5 g) and triethylamine (3.5 ml) were added. After 48 hr, the reaction mixture was dissolved in ethyl acetate and washed successively with 1 *N* NH_4OH, water, 0.5 *N* HCl, and water. The material obtained after evaporation of the solvent was dissolved in ethanol (100 ml) and precipitated by the addition of water (300 ml). The precipitated product was dissolved in hot methanol (70 ml) and precipitated upon dilution of this solution with water (400 ml): 14.4 g (85%); mp 174–176°; $[\alpha]^{26}D$ −13.5° (*c* 1, DMF). After hydrogenolysis in the presence of HCl: R_f^1 0.82, R_f^2 3.12 × His. Amino acid analysis after acid hydrolysis: $Gly_{1.0}Tyr_{0.8}Phe_{2.0}$.

Anal. Calcd for $C_{38}H_{40}N_4O_8$: C, 67.0; H, 5.92; N, 8.2. Found: C, 66.9; H, 6.09; N, 8.3.

N^α-Benzyloxycarbonyl-N^ω-tosyl-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosine Methyl Ester (IV). Compound III (6 g) was hydrogenated for 3.5 hr over palladium/charcoal catalyst (1 g) in methanol (150 ml) containing 2 *N* HCl (4.6 ml). The catalyst was filtered off and the solvent was removed under reduced pressure. The residue was dried by repeated addition and evaporation of methanol under reduced pressure and used for condensation with N^α-benzyloxycarbonyl-N^ω-tosyl-L-arginine.[20] A solution of the latter compound (4.5 g) in acetonitrile (125 ml) and DMF (10 ml) was cooled to 0° and triethylamine (1.35 ml) was added followed by 2-ethyl-5-phenylisoxazolium 3′-sulfonate (2.45 g). After 1 hr at 0° the reaction mixture was mixed with a solution of the tetrapeptide ester in DMF and acetonitrile prepared as follows. The hydrochloride salt, which had been prepared as described previously, was dissolved in a cold (0°) mixture of DMF (30 ml) and acetonitrile (40 ml) containing triethylamine (1.25 ml), stirred 5 min, and then added to the activated N^α-benzyloxycarbonyl-N^ω-tosyl-L-arginine prepared as described above. After 48 hr at room temperature, the reaction mixture was poured into 1 *N* $NaHCO_3$ (600 ml; 0°) saturated with NaCl. The precipitated semisolid product was extracted into ethyl acetate (600 ml) and the organic phase was washed successively with 0.5 *N* NH_4OH, water, 0.5 *N* HCl, and water. The white solid obtained after removal of the ethyl acetate was dissolved in methanol (60 ml) precipitated by the addition of saturated aqueous NaCl, isolated by filtration, and washed with cold water: 7.1 g (81%); melting point undetermined; the peptide sinters at 112° and it is converted to liquid at 135°; $[\alpha]^{26}D$ −10.3° (*c* 1, DMF). After hydrogenolysis in the presence of HCl: R_f^1 0.90, R_f^2 3.80 × His. Amino acid analysis after acid hydrolysis: $Arg_{1.0}Gly_{1.0}Tyr_{0.9}Phe_{2.0}$.

Anal. Calcd for $C_{51}H_{58}N_8O_{11}$: C, 61.8; H, 5.90; N, 11.3. Found: C, 61.3; H, 6.10; N, 11.3.

N-Benzyloxycarbonyl-γ-*tert*-butyl-L-glutamyl-N^ω-tosyl-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosine Methyl Ester (V). The protected pentapeptide ester IV (5.4 g) was hydrogenated for 4 hr over 10% palladium/charcoal catalyst (1.5 g) in methanol (150 ml) containing 2 *N* HCl (2.4 ml). The catalyst was filtered off and the filtrate was concentrated to dryness under reduced pressure. The residue which was dried by repeated addition and evaporation of methanol under reduced pressure was dissolved in DMF (60 ml). To this solution cooled to 0°, triethylamine (0.63 ml) was added followed by *N*-benzyloxycarbonyl-L-glutamic acid γ-*tert*-butyl-α-*p*-nitrophenyl ester[23] (2.17 g). After 48 hr the reaction mixture was poured into ethyl acetate (400 ml) and water (100 ml) and the organic phase was washed successively with 0.5 *N* NH_4OH, water, 0.2 *N* H_2SO_4, and water. The solid obtained after evaporation of the solvent under reduced pressure was dissolved in methanol (60 ml) and precipitated by the addition of ether (170 ml): 3.9 g (78%); melting point undetermined; the peptide sinters at 108° and it is converted to liquid at 126°; $[\alpha]^{26}D$ −10.9° (*c* 1, DMF). After hydrogenolysis in the presence of acetic acid: R_f^1 0.93, R_f^2 4.06 × His. Amino acid analysis of an acid hydrolysate: $Arg_{1.0}Glu_{1.0}Gly_{1.0}Tyr_{0.7}Phe_{2.0}$.

Anal. Calcd for $C_{60}H_{73}N_9O_{14}S$: C, 61.3; H, 6.26; N, 10.7. Found: C, 61.4; H, 6.59; N, 10.8.

N-Benzyloxycarbonyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-*S*-benzyl-L-cysteinylglycine (VI). This compound was prepared by saponification of the corresponding ethyl ester[37] in essentially the same way described for the saponification of the methyl ester derivative.[38]

N-Benzyloxycarbonyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-*S*-benzyl-L-cysteinylglycyl-γ-*tert*-butyl-L-glutamyl-N^ω-tosyl-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosine Methyl Ester (VII). The protected hexapeptide ester V (1.77 g) was hydrogenated for 4 hr over 10% palladium/charcoal catalyst (0.5 g) in methanol (60 ml) containing glacial acetic acid (0.5 ml). The catalyst was filtered off and the filtrate was concentrated to dryness under reduced pressure. The residue (Va) was dried by repeated addition and evaporation of methanol under reduced pressure and used for condensation with the partially protected hexapeptide VI. A solution of VI (1.49 g) and *N*-hydroxysuccinimide (0.19 g) in DMF (8 ml) was cooled to −10° and *N*,*N*′-dicyclohexylcarbodiimide (0.34 g) was added. After 12 hr at 5°, the reaction mixture was filtered through a sintered-glass funnel and the filtrate was mixed with the solution of the hexapeptide ester in DMF prepared as follows: the acetate of the hexapeptide ester, which had been prepared as described previously, was dissolved in DMF (7 ml) containing triethylamine (0.18 ml), stirred 2 min at 0°, and added to the reaction mixture prepared as described above. After 24 hr at room temperature, the reaction mixture was poured into methanol (300 ml). The precipitated product was filtered off, washed with methanol, dried, and reprecipitated from dimethylformamide–methanol: 2.2 g (88%); mp 254–255°; $[\alpha]^{26}D$ −24.9° (*c* 1, DMF).

Anal. Calcd for $C_{98}H_{127}N_{13}O_{21}S_2$: C, 61.5; H, 6.68; N, 11.0. Found: C, 61.1; H, 6.58; N, 10.8.

Amino acid analysis of an acid hydrolysate of the protected

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dodecapeptide showed the expected composition expressed in molar ratios: $Arg_{0.9}Glu_{1.0}Gly_{2.0}Val_{1.0}Leu_{2.1}Tyr_{1.7}Phe_{1.9}$*S*-benzylcysteine$_{0.9}$.

N^{α}-Benzyloxycarbonyl-*im*-benzyl-L-histidyl-L-leucyl-L-valyl-γ-*tert*-butyl-L-glutamyl-L-alanine (VIII). To a suspension of *N*-benzyloxycarbonyl-*im*-benzyl-L-histidyl-L-leucyl-L-valyl-γ-*tert*-butyl-L-glutamyl-L-alanine methyl ester[39] (8.87 g) in acetone (150 ml) cooled to 5° was added over a period of 30 min 1 *N* NaOH (20.6 ml) and water (17 ml). The reaction mixture was further stirred at room temperature for 30 min, cooled to 5°, and diluted with 2 *N* HCl (10.3 ml) and water (150 ml). The precipitated product was isolated by filtration, washed thoroughly with water, dried, and crystallized from a solution in methanol (250 ml) by the addition of water (18 ml): 7.6 g (87%); mp 251–252°; $[\alpha]^{26}D$ −18.3° (*c* 1, DMF). After HBr in acetic acid treatment, and on thin-layer chromatography (Eastman Organic chromagram, Silica Gel 6060, solvent systems: 1-butanol–water–acetic acid (4:1:1); 1-butanol–acetic acid–water–pyridine (30:6:24:20)), the peptide derivative exhibited a single spot.

Anal. Calcd for $C_{44}H_{61}N_7O_{10}$: C, 62.3; H, 7.24; N, 11.5. Found: C, 62.3; H, 7.36; N, 11.6.

Amino acid analysis, after acid hydrolysis, showed the following composition expressed in molar ratios: $Glu_{1.0}Ala_{1.0}Val_{1.0}Leu_{1.0}$-benzylhistidine$_{1.0}$. For evaluation of stereochemical homogeneity, a sample of the deblocked (HBr in acetic acid) derivative was digested with APM. Amino acid analysis of the digest gave the following amino acid composition expressed in molar ratios: $Glu_{1.0}Ala_{1.0}Val_{0.9}Leu_{1.0}$benzylhistidine$_{0.9}$.

N^{α}-Benzyloxycarbonyl-*im*-benzyl-L-histidyl-L-leucyl-L-valyl-γ-*tert*-butyl-L-glutamyl-L-alanyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-*S*-benzyl-L-cysteinylglycyl-L-glutamyl-N^{ω}-tosyl-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosine Methyl Ester (IX). The protected dodecapeptide VII (1.92 g) was dissolved in trifluoroacetic acid (20 ml) containing water (0.2 ml) and hydrogen bromide was passed through the solution for 1 hr at room temperature. After most of the trifluoroacetic acid was removed under reduced pressure, the remaining product was mixed with anhydrous ether (100 ml). The precipitated dodecapeptide ester hydrobromide (VIIa) was isolated by centrifugation, washed thoroughly with ether, and dried over KOH *in vacuo*, 1.86 g. To a solution of this product in DMF (20 ml), cooled to 0°, triethylamine (0.28 ml) was added followed by the partially protected pentapeptide VIII, which has been activated as follows. A solution of VIII (1.7 g) and *N*-hydroxysuccinimide (0.23 g) in DMF (35 ml) was cooled to −10° and *N*,*N*′-dicyclohexylcarbodiimide (0.41 g) was added. After 1 hr at −10° and 12 hr at 5°, the reaction mixture was filtered with the aid of a sintered-glass funnel and the filtrate added to the solution of the dodecapeptide ester prepared as described above. After 48 hr at room temperature the reaction mixture was poured into methanol (400 ml) containing acetic acid (3 ml). The precipitated product was isolated by centrifugation, triturated with hot methanol (140 ml) and water, and dried: 2.45 g (90%); mp 264–265°; $[\alpha]^{26}D$ −18.2° (*c* 1, DMSO).

Anal. Calcd for $C_{130}H_{173}N_{22}O_{28}S_2H_2O$: C, 60.6; H, 6.85; N, 12.0. Found: C, 60.2; H, 7.02; N, 11.9.

Amino acid analysis of an acid hydrolysate of the protected heptadecapeptide showed the expected composition expressed in molar ratios: $Arg_{1.0}Glu_{2.0}Gly_{1.9}Ala_{1.0}Val_{2.0}Leu_{3.0}Tyr_{1.4}Phe_{2.0}$benzylhistidine$_{1.0}$*S*-benzylcysteine$_{0.7}$.

***N*-*tert*-Butoxycarbonyl-*O*-benzyl-L-seryl-*im*-benzyl-L-histidyl-L-leucyl-L-valyl-L-glutamyl-L-alanyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-*S*-benzyl-L-cysteinylglycyl-L-glutamyl-N^{ω}-tosyl-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosine (X).** The heptadecapeptide derivative IX (1 g) was dissolved in cold trifluoroacetic acid (15 ml) containing water (0.2 ml) and hydrogen bromide was passed through the solution for 60 min at room temperature. After most of the trifluoroacetic acid was removed under reduced pressure, the remaining product was mixed with anhydrous ether. The precipitated heptadecapeptide ester hydrobromide (IXa) was washed several times with ether, reprecipitated from a suspension in 2-propanol by the addition of ether, and dried over KOH *in vacuo*, 0.99 g. This solid was dissolved in hexamethylphosphoramide (20 ml), cooled to 0°, and then neutralized with triethylamine (0.16 ml) just prior to the addition of the activated *N*-*tert*-butoxycarbonyl-*O*-benzyl-L-serine prepared as follows: *N*-*tert*-butoxycarbonyl-*O*-benzyl-L-serine (1.53 g) and *N*-hydroxysuccinimide (0.59 g) were dissolved in DMF (15 ml), and to this solution cooled to −10° was added *N*,*N*′-dicyclohexylcarbodiimide (1.05 g). After 1 hr at −10° and 3 hr at 5°, the precipitated *N*,*N*′-dicyclohexylurea was filtered off and the filtrate was added to the solution of the heptadecapeptide ester prepared as described above. After 24 hr at room temperature the reaction mixture was poured into methanol (500 ml). The precipitated product was filtered off and washed with very dilute acetic acid and water, 0.68 g, mp 270–273°. The protected octadecapeptide (Xa) was subsequently dissolved in hexamethylphosphoramide (12 ml) with stirring and warming to 40°. To this solution, cooled to 5°, was added portionwise and over a period of 30 min 1 *N* NaOH (1.8 ml) and water (4 ml). The reaction mixture was stirred for an additional 20 min at room temperature and subsequently was cooled to 0° and diluted with cold water (150 ml) and 2 *N* HCl (0.9 ml). The precipitated white solid was isolated, washed with water, and dried: 0.59 g (57%, based on the amount of VII used); mp 273–276°; $[\alpha]^{26}D$ −17.7° (*c* 1, DMSO).

Amino acid analysis of an acid hydrolysate of the partially protected octadecapeptide showed the expected composition expressed in molar ratios: $Arg_{1.0}Ser_{0.8}Glu_{2.1}Gly_{2.1}Ala_{1.0}Val_{1.9}Leu_{2.9}Tyr_{1.5}Phe_{2.1}$benzylhistidine$_{0.9}$*S*-benzylcysteine$_{0.7}$.

L-Phenylalanyl-L-valyl-L-asparaginyl-L-glutaminyl-L-histidyl-L-leucyl-*S*-sulfo-L-cysteinylglycyl-L-seryl-L-histidyl-L-leucyl-L-valyl-L-glutamyl-L-alanyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-*S*-sulfo-L-cysteinylglycyl-L-glutamyl-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosine (Destetrapeptide B^{27-30} Chain *S*-Sulfonate) (XI). A solution of the octadecapeptide derivative X (0.64 g) in trifluoroacetic acid (10 ml) was stored at room temperature for 1 hr and then diluted with ether. The precipitated partially deblocked octadecapeptide, *O*-benzyl-L-seryl-*im*-benzyl-L-histidyl-L-leucyl-L-valyl-L-glutamyl-L-alanyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-*S*-benzyl-L-cysteinylglycyl-L-glutamyl-N^{ω}-tosyl-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosine trifluoroacetate (Xb) was isolated, washed with ether, and dried over KOH *in vacuo*. This material was dissolved in hexamethylphosphoramide (15 ml) with stirring and warming to 40°. This solution cooled to 0°, was neutralized with triethylamine (0.15 ml) just prior to the addition of the N-terminal octapeptide derivative which was activated as follows. *N*-Benzyloxycarbonyl-L-phenylalanyl-L-valyl-L-asparaginyl-L-glutaminyl-*im*-benzyl-L-histidyl-L-leucyl-*S*-benzyl-L-cysteinylglycine (Xc; 1.0 g) and *N*-hydroxysuccinimide (90 mg) were dissolved in a mixture of DMF (10 ml) and hexamethylphosphoramide (7 ml) and to this solution cooled to −5° was added *N*,*N*′-dicyclohexylcarbodiimide (167 mg). After 12 hr at 5° the reaction mixture was added to the solution of the partially protected octadecapeptide prepared as described above. The reaction mixture was stirred at room temperature for 40 hr, diluted with acetic acid (0.4 ml), and poured into methanol (350 ml). The precipitated product was isolated by centrifugation, washed successively with warm methanol and ether, and dried, 476 mg. An acid hydrolysate of this material showed the following composition in molar ratios: $Arg_{0.9}Asp_{0.9}Ser_{0.7}Glu_{3.0}Gly_{3.2}Ala_{0.9}Val_{2.9}Leu_{4.0}Tyr_{1.3}Phe_{2.6}$benzylhistidine$_{2.0}$*S*-benzylcysteine$_{1.5}$. The crude protected hexacosapeptide (XIa) was converted to the S-sulfonated derivative (destetrapeptide B^{27-30} chain *S*-sulfonate) by removing the protecting groups with sodium in liquid ammonia and sulfitolyzing the resulting product, with sodium sulfite and sodium tetrathionate. The reduction procedure was essentially that described in the synthesis of the B-chain *S*-sulfonate of sheep and human insulin[15,16] and was carried out in the glass apparatus devised for sodium–liquid ammonia reactions.[16] Briefly, the crude protected hexacosapeptide (200 mg) was dissolved in anhydrous liquid ammonia (200 ml) in a 500-ml round-bottomed flask fitted for magnetic stirring. The reduction was carried out at the boiling point of the solution by the dropwise addition of a liquid ammonia solution of sodium into the reaction mixture. The faint blue color, indicating an excess of sodium at the end point of the sodium in liquid ammonia reaction, was allowed to persist for 20 sec and then discharged by the addition of a few crystals of ammonium chloride. The resulting clear solution was concentrated at atmospheric pressure to about 10 ml and dried from the frozen state. The residue was dissolved in 8 *M* guanidine hydrochloride (30 ml) and the pH of the solution was adjusted to 8.9 with acetic acid or dilute NH_4OH (depending on the pH of the solution). To this solution was added freshly prepared sodium tetrathionate (250 mg) and, 30 min later, sodium sulfite (500 mg). The reaction mixture was stirred for 3 hr at room temperature and then placed in an 18/32 Visking dialysis tubing and dialyzed against four changes of distilled water (4 l. each) at 4° for 24 hr. Lyophilization of the dialyzate gave the

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crude destetrapeptide B^{27-30} chain *S*-sulfonate as a white fluffy solid, 145 mg. Amino acid analysis of this material after acid hydrolysis gave the following composition expressed in molar ratios: $His_{1.8}Arg_{0.7}Asp_{0.9}Ser_{6.8}Glu_{3.0}Gly_{3.1}Ala_{1.2}Cys_{1.6}Val_{3.1}Leu_{4.4}Tyr_{1.3}$-$Phe_{2.6}$.

Isolation of Destetrapeptide B^{27-30} Chain *S*-Sulfonate. The lyophilized crude product (72 mg) was dissolved in 5 ml of urea–acetate buffer (0.04 *M* sodium acetate–8 *M* urea, pH 4.0) and applied to a CM-cellulose column (4 × 55 cm) equilibrated with the same buffer. The preparation of the column has been described in previous reports.[12,13] The chromatogram was developed with the aforementioned buffer at a flow rate of 120 ml/hr, and the eluted material was monitored continuously with a Gilford recording spectrophotometer at 278 mμ. The chromatographic pattern obtained (Figure 2-II) indicated the presence of three main components 1, 2, and 3. Figure 2-I illustrates the chromatographic profile of natural bovine B chain *S*-sulfonate in the same chromatographic system. Identification of components 1 and 2 was not attempted. For removing the urea, the effluent containing component 3 (500–590 ml of effluent) was adjusted to pH 3 with concentrated HCl and placed on a Sephadex G-15 column (4 × 60 cm) equilibrated and eluted with 5% (v/v) acetic acid. The pooled fractions containing the peptide material (detected with a Gilford recording spectrophotometer at 278 mμ) were concentrated in a rotary evaporator (30°) to about 10 ml and mixed with 12 ml of saturated picric acid solution. After 24 hr at 2° the precipitated picrate of destetrapeptide B^{27-30} chain *S*-sulfonate was isolated by centrifugation and washed with half-saturated picric acid solution. Separation of the picric acid from the peptide chain was accomplished upon chromatography of its picrate on a 1.2 × 50 cm Sephadex G-15 column equilibrated with 0.05 *N* NH_4HCO_3. The picrate of the destetrapeptide B^{27-30} chain was dissolved in 0.05 *N* NH_4HCO_3 (2 ml) containing a few drops of 1 *N* NH_4OH, placed on the column and eluted with 0.05 *N* NH_4HCO_3.[12] The peptide material, located by continuous monitoring of the effluent with a Gilford recording spectrophotometer at 278 mμ, was recovered as a white fluffy material by lyophilization of the effluent, wt 10 mg. Amino acid analysis of the purified material after acid hydrolysis gave the molar ratios of amino acids shown in Table I in very good agreement with the theoretically expected values. Digestion of the synthetic material with APM and amino acid analysis of the digest gave the amino acid ratios shown in Table I in good agreement with the theoretically expected values. On thin-layer electrophoresis in 0.01 *M* NH_4HCO_3 (adjusted to pH 10.0 with NH_4OH) and 2800 V the synthetic chain analog moved as a single component (Pauly reaction) and had a greater mobility toward the anode than the natural B-chain *S*-sulfonate (Figure 3).

***S*-Sulfonated Derivative of the A Chain of Human (Porcine) Insulin.** This compound was prepared by oxidative sulfitolysis of porcine insulin by the method which has been described in a previous report.[12]

Isolation of the Insulin Analog Produced by Combination of Synthetic Destetrapeptide B^{27-30} Chain and Natural Human (Porcine) A Chain (Destetrapeptide B^{27-30} Human (Porcine) Insulin). The synthesis of this insulin analog was carried out by the interaction of the sulfhydryl form of the A chain with the sulfonated form of the destetrapeptide B^{27-30} chain according to the procedure described previously.[13,24] In a typical combination experiment, an aqueous solution of 20 mg of S-sulfonated human (porcine) A chain was reacted at pH 5.0 with 2-mercaptoethanol for 6 min at 100°. After cooling to 5°, the reaction mixture was extracted three times with ethyl acetate to remove the 2-mercaptoethanol and the resulting sulfhydryl form of the A chain was allowed to react with 5 mg of S-sulfonated destetrapeptide B^{27-30} chain for 20 hr at pH 9.6 and 0°. The reaction mixture (10 ml) was subsequently diluted with acetic acid (1 ml) and mixed with an equal volume of saturated picric acid solution and stored for 24 hr at 2°. The precipitated picrate was collected by centrifugation, washed twice with 5-ml portions of a half-saturated picric acid solution, and dissolved in a few drops of acetone–water (4:1, v/v). Addition of cold, dry acetone (40 ml), containing 3–4 drops of concentrated HCl, to this solution caused the precipitation of the product as the hydrochloride. After 24 hr at 2° the precipitated material was isolated by centrifugation and washed with acetone and ether. This precipitate consists of the various products formed by the combination of the A and destetrapeptide B^{27-30} chains. Separation of the insulin analog from this mixture was accomplished by chromatography on a CM-cellulose column with an exponential NaCl gradient as was described in detail previously.[13,25] Briefly, a suspension of the above-mentioned precipitate in 1 ml of acetate buffer (pH 3.3, Na^+, 0.024 *M*) was stirred for 1 min and the insoluble material was removed by centrifugation and washed with 2 ml of the same buffer. The supernatant and washings were combined. One more combination mixture was processed separately up to this point. The combined supernatants were placed on a 0.9 × 23 cm CM-cellulose column and chromatographed as described previously.[13,25] The chromatographic pattern obtained is shown in Figure 4. The insulin analog is eluted with application of the gradient and is the slowest moving material. The effluent containing the insulin analog (130–150 ml of effluent) was concentrated in a rotary evaporator (20–25°) to approximately 10 ml and mixed with 12 ml of saturated picric acid solution. After 24 hr at 2°, the precipitated picrate of the destetrapeptide B^{27-30} human (porcine) insulin was isolated by centrifugation, washed once with one-half saturated picric acid solution (2 ml), and converted to the hydrochloride,[25] wt 0.3 mg.

Amino acid analysis of this analog after acid hydrolysis gave a composition expressed in molar ratios shown in Table II, in good agreement with the theoretically expected values. On thin-layer electrophoresis in 0.5 *N* acetic acid at 3400 V, the synthetic analog (Figure 5) moved as a single component (Pauly reaction). The destetrapeptide B^{27-30} human (porcine) insulin by the mouse convulsion assay method was found to possess a potency of 11–15 IU/mg and by radioimmunoassay had a potency of 7 IU/mg.

Table II. Amino Acid Composition[a] of an Acid Hydrolysate of the Destetrapeptide B^{27-30} Human (Porcine) Insulin

Amino acid	Theory	Found
Histidine	2	2.2
Arginine	1	1.1
Aspartic acid	3	2.7
Threonine	1	0.8
Serine	3	2.4[b]
Glutamic acid	7	6.7
Glycine	4	4.2
Alanine	1	1.2
Half-cystine	6	4.0[b]
Valine	4	3.7
Isoleucine	2	1.3
Leucine	6	5.7
Tyrosine	4	3.2[b]
Phenylalanine	3	3.1

[a] Number of amino acid residues per molecule. [b] Uncorrected for destruction.

The component eluted from the CM-cellulose column just before the insulin analog (100–120 ml of effluent, Figure 4) was isolated *via picrate* as the hydrochloride following the procedure employed for the isolation of the analog. Amino acid analysis after acid hydrolysis gave the following composition expressed in molar ratios: $His_{1.9}Arg_{1.0}Asp_{1.1}Ser_{0.9}Glu_{3.1}Gly_{3.0}Ala_{1.0}Cys_{1.8}Val_{3.0}Leu_{4.0}Tyr_{1.6}$-$Phe_{2.9}$. This composition is in excellent agreement with the theoretically expected values for the destetrapeptide human B^{27-30}f chain.

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